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II. REMARKS

Preliminary Remarks

Reconsideration and allowance of the present application based on the following remarks are respectfully requested. Claims 20-36 are currently pending in this application. Claims 20, 23, 30, and 33-36 have been allowed. Claims 21, 22, 24-29, 31, and 32 remain at issue.

New claims 37-44 are directed to vectors comprising the nucleic acid of claims 21, 22, or 31, and host cells comprising such vectors. Support can be found throughout the specification, for example, on pages 3 and 4 and originally filed claims 8 and 9.

The applicants do not intend by these or any amendments to abandon subject matter of the claims as originally filed or later presented, and reserve the right to pursue such subject matter in continuing applications.

Patentability Remarks

Rejection Pursuant to 35 U.S.C. §112, First Paragraph, Enablement

Claims 21, 22, 24, 25, 28, and 29

In paragraph 4 of the official action, the examiner rejected claims 21, 22, 24, 25, 28, and 29 under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement. Specifically, the examiner alleged the specification does not reasonably provide enablement to any polynucleotide that is at least 90% or 95% identical to a polynucleotide encoding a polypeptide that has the amino acid sequence of SEQ ID NO: 2 or polynucleotide of SEQ ID NO: 1. The examiner further asserted that while the specification teaches how to search and identify the claimed polynucleotides, the specification does not teach how to make the claimed polynucleotides. The examiner further alleged the specification does not teach the specific structural/catalytic amino acids and the structural motifs essential for protein activity/function which must be preserved. The examiner concludes that without such guidance, the experimentation left to those skilled in the art is undue.

Amended claims 21 and 22 are directed to an isolated nucleic acid encoding a polypeptide that is 90% or 95% identical to the amino acid sequence of SEQ ID NO: 2 having the same activity as O-succinylbenzoic acid CoA ligase. Support for amended claims 21 and

22 can be found throughout the specification, for example, page 3, lines 4 and 5, page 6, lines 21-23, and page 4, line 31 to page 5, line 3.

The issue of enablement involves the question of whether an application enables one of ordinary skill in the art to make and to use the claimed invention. Experimentation is simply limited in amended claims 21 and 22 to identifying nucleic acids encoding a polypeptide that is 90% or 95% identical to the amino acid sequence of SEQ ID NO: 2 having the same activity as O-succinylbenzoic acid CoA ligase. Therefore, it is the applicants' position that there is no question that the variants encompassed by the claims must retain the utility of the DNA sequence discovered and claimed by the applicants. The applicants further submit the specification enables one of skill in the art to make and functionally define the claimed variants.

Specifically, claims 21 and 22 satisfy the "how to make" prong of the enablement requirement because the scope of the claim is "reasonably correlated" with the teachings in the application [See MPEP §2164.01(b)]. The application and ordinary skill permit one skilled in the art to make any polynucleotide having 90% or greater sequence identity to the sequence recited in the claims using routine PCR technology or other routine mutagenesis techniques discussed on page 8, lines 8-20 and 29-35 and page 10, line 16 to line 31. In fact, the Patent Office's own training materials acknowledge that "procedure[s] for making variants of [a polypeptide having] SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3: and retain its activity are conventional in the art." (See Revised Interim Written Description Guidelines Training Materials, Example 14). Moreover, the present application provides guidance as to the types of changes (*e.g.*, conservative mutations) that are more likely to retain functionality (see specification, page 8, lines 12-28).

In addition, the variants defined by claims 21 and 22 are also functionally defined in that the claimed variants are limited to those with the same activity as O-succinylbenzoic acid CoA ligase (*i.e.*, enzymatic conversion of the benzenoid aromatic compound o-succinylbenzoic acid [OSB; 4-(2'-carboxyphenyl)-4-oxobutyric acid] to the naphthalenoid aromatic compound 1,4-dihydroxy-2-naphthoic acid [DHNA]). The specification specifically refers to the inventors successfully isolating the novel *men*E gene from *C. glutamicum* (Example 2). The specification also specifically describes that the variant polypeptide with at least 90-95% identity to SEQ ID NO: 2 must have the same activity as O-succinylbenzoic acid CoA ligase (page 5, lines 7-13).

The disclosure teaches the full length *men*E gene has been isolated from *C. glutamicum* and using PCR technology, the whole gene, or fragments thereof, can be cloned into expression vectors (see Examples 2 and 3). The full length *men*E gene has also been isolated, sequence, and cloned into expression vectors in *E. coli* and the gram positive bacterium *Micrococcus phlei* (see Appendix A, Kwon *et al., J. of Bact.* 178:6778-6781 (1996), Meganathan *et al., J. Bacteriol.* 140:92-98 (1979), and Sieweke *et al., Z. Naturforsch* 46c:585-590 (1991) (abstract only)).

Two different assays have been designed for *Micrococcus phlei* and *E.coli* which measure the activity of O-succinylbenzoic acid CoA ligase. These screening assays were well known in the art at the time of filing and would allow one of skill to identify those encoded polypeptides with the O-succinylbenzoic acid CoA ligase activity as required by the claims (Meganathan *et al.*, at pg. 93, first column and Figure 2; Kwon *et al.*, pg. 6779 first column and pg. 6780). The substrate O-malonylbenzoic acid was found to be a suitable substrate for either assay discussed in Meganathan *et al.* or Kwon *et al.* and thus, one of skill could perform this assay without undue experimentation.

In addition, in view of these teachings, the examiner's point regarding the lack of correlation between specific amino acid residues and activity/function of O-succinylbenzoic acid CoA ligase taught in the specification, is not applicable in this situation. Variants that do not have a functional *men*E gene product can be easily identified from *men*E variants that are able to produce a polypeptide that maintains O-succinylbenzoic acid CoA ligase activity.

Thus, in view of the teachings of the present specification and what was known in the art at the time of filing, the experimentation necessary to practice the present invention would not be undue. Accordingly, the structural and catalytic properties of *men*E were well known to those skill in the art at the time of filing and thus, the specification's requirement that the variants maintain O-succinylbenzoic acid CoA ligase activity is fully enabled by the specification.

Solely for the purpose of expediting prosecution, and without prejudice to the applicants' right to seek broader claims in a continuing application, the applicants have amended claim 24 to be directed to a vector comprising the nucleic acid of claim 20 or claim 23. Since claims 20 and 23 have been allowed, the claims 24, 25, 27, and 28 are now in a condition for allowance as well. New claims 37-40 are ultimately dependent upon amended claims 21 and 22 (*e.g.*, vectors comprising the nucleic acids of claims 21 and 22, host cell comprising the vectors) and are fully described in the specification.

In view of the foregoing amendments and remarks, the applicants submit that the rejection of claims 21 and 22 pursuant to 35 U.S.C. §112, first paragraph, for lack of enablement, has been overcome and should be withdrawn, and a rejection of new claims 37-40 on the same grounds would be improper.

Claims 31 and 32

In paragraph 6 of the official action, the examiner rejected claims 31 and 32 under 35 U.S.C. §112, first paragraph. Specifically, the examiner alleged claims 31 and 32 encompass any nucleic acid of any nucleotide sequence consisting of at least 40 consecutive nucleotides of SEQ ID NO: 1, wherein the nucleic acid functions as a primer or probe. The examiner further asserted the specificity of hybridization probes is empirical by nature and the effect of mismatches within an oligonucleotide probe is unpredictable. The examiner alleged that even if the probe is a 20-mer, the total number of hits in a database search was 143,797,728, which suggest that some of the probes encompassed by claims 31 and 32 would not preferentially hybridize to SEQ ID NO: 1. The examiner concluded by stating the predictability of which oligonucleotides or probes hybridize specifically to SEQ ID NO: 1 would require undue experimentation of one skilled in the art to practice the claimed invention.

Amended claim 31 is directed to an isolated nucleic acid consisting of a fragment of at least 40 consecutive nucleotides of SEQ ID NO: 1 or the full complement thereof, wherein said isolated nucleic acid is a probe in a hybridization reaction to detect an isolated nucleic acid that is at least 90% identical to that of SEQ ID NO: 1 and encodes a polypeptide that has O-succinylbenzoic acid CoA ligase activity and wherein said hybridization reaction comprise the following stringent conditions: a final wash in 0.1 SSCX at 68°C. Support for amended claim 31 can be found throughout the specification, for example, on pages 4, lines 22-26 and page 9, lines 1-25.

The applicants respectfully submit claim 31 is directed to a unique and finite set of nucleic acids and would not require undue experiementation by one of skill in the art to practice the claimed invention. The specific criteria for the nucleic acids of claim 31 is as follows: (a) the nucleic acid must consist of a fragment of at least 40 consecutive nucleotides of SEQ ID NO: 1; (b) the nucleic acid is used as a probe in a hybridization reaction **to detect an** isolated nucleic acid that is at least 90% identical to that of SEQ ID NO: 1 and encodes a polypeptide that has O-succinylbenzoic acid CoA ligase activity. The hybridization reaction

using the nucleic acids of claim 31 must also include a final wash in 0.1X SSC at 68°C. In view of the fact (and as noted on numerous occasions by the federal judiciary), the level of skill in molecular biology arts is high and therefore in view of the requirements of claim 31, one in this field could easily predict the number of nucleic acids encompassed by claim 31 without due experimentation. In view of the foregoing amendments and remarks, the applicants submit that the rejection of claims 31 and 32 pursuant to 35 U.S.C. §112, first paragraph, for lack of enablement, is overcome and should be withdrawn.

Rejection Under 35 U.S.C. §112, First Paragraph, Written Description

In paragraph 5 of the official action, the examiner rejected claim 26 under 35 U.S.C. §112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, has possession of the claimed invention. Specifically, the examiner alleged the specification does not provide written description of any vector that is an integration vector pCR.1menEint of any nucleotide sequence having an internal 520 bp fragment of any *men*E gene of any nucleotide sequence. The examiner asserted that the specification, however, only teaches a vector deposited in *E. coli* strain Top10/pCR2.1menEint under Accession No. DSM14080. The examiner further alleged the specification does not provide a written description of any vector that is an integration vector pCR.1menEint of any nucleotide sequence having an internal 520bp fragment of any *men*E gene of any nucleotide sequence.

Amended claim 26 is directed to the vector of claim 25 that is an integration vector pCR.1menEint having an internal fragment of SEQ ID NO: 1 and a restriction map as set forth in Figure 1. The applicants submit amended claim 26 is directed to a specific integration vector. Claim 26 requires the intergration vector pCR.1menEint to contain a particular nucleotide sequence. This nucleotide sequence is an internal fragment of SEQ ID NO: 1, which is the *men*E gene isolated from *C. glutamicum* (see Example 1). Claim 26 also requires the integration vector have the restriction map set forth in Figure 1 which was constructed as described in Example 3. Figure 1 shows the restriction map of pCR.1menEint. The restriction map indicates pCR.1menEint is 4.47kb, and contains a gene encoding for kanamycin resistance, the *men*E gene isolated from *C.glutamicum*, and the genetic component ColE1, which controls replication. Accordingly, a skilled artisan would recognize the applicants were in possession of the claimed invention from the teachings of the specification

(for example Example 3 and Figure 1). In view of the foregoing amendment and remarks, the applicants submit that the rejection of claim 26 under 35 U.S.C. §112, first paragraph, written description, has been overcome and should be withdrawn.

Rejection Under 35 U.S.C. §112, Second Paragraph, Indefiniteness

In paragraph 8 of the official action, the examiner rejected claims 21 and 22 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite. Specifically, the examiner asserted the phrase "identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID NO: 2" is vague because the specific nucleotide sequence to which the claimed polynucleotide has 70% identity is not known or defined in the specification. The examiner further alleged that the phrase "the polypeptide preferably having the activity of O-succinylbenzoic acid CoA ligase" is vague because it is not known if the claimed polynucleotide has the recited activity.

As stated above, amended claims 21 and 22 are directed to an isolated nucleic acid encoding a polypeptide that is 90% or 95% identical to the amino acid sequence of SEQ ID NO: 2 having the same activity as O-succinylbenzoic acid CoA ligase. The applicants respectfully submit that claims 21 and 22 are now directed to the specific nucleic acid sequence encoding a polypeptide of the amino acid sequence SEQ ID NO: 2 which is defined in the specification. In addition, claims 21 and 22 define the activity of SEQ ID NO: 2 as being an amino acids sequence that encodes a polypeptide that has O-succinylbenzoic acid CoA ligase activity. In view of the foregoing amendments and remarks, the applicants submit that the rejection of claims 21 and 22 under 35 U.S.C. §112, second paragraph, for indefiniteness, is overcome and should be withdrawn.

CONCLUSION

In view of the foregoing, the claims are now believed to be in form for allowance, and such action is hereby solicited. If any point remains in issue which the Examiner feels may be best resolved through a personal or telephone interview, please contact the undersigned at the telephone number listed below.

All objections and rejections having been addressed, it is respectfully submitted that the present application is in a condition for allowance and a Notice to that effect is earnestly solicited.

Respectfully submitted,

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

Applicant: FARWICK et al.

Appln. No.: 09/834,722

Filing Date: April; 16, 2001

Date: January 2, 2004 Page 1 of 1

Examiner: C. Fronda | Group Art Unit: 1652

U.S. PATENT DOCUMENTS

Examiner's Initials*		Document Number	Date MM/YYYY	Name (Family Name of First Inventor)	Class	Sub Class	Filing Date (if appropriate)
	AR						
	BR						
	CR						
	DR						
	ER						
	FR						
	GR						
	HR						
	IR						
	JR						
	KR						

FOREIGN PATENT DOCUMENTS

		Document Number	Date MM/YYYY	Country	Inventor Name		English Abstract		Translation Readily Available	
							Enclosed	No	Enclose	No
	LR									
	MR									
	NR									
	OR									
	PR									
	QR									
	RR									

OTHER (Including in this order Author, Title, Periodical Name, Date, Pertinent Pages, etc.)

	SR	Sieweke HJ et al. (1991) "o-Succinylbenzoate: coenzyme A ligase, an enzyme involved in menaquinone (vitamin K2) biosynthesis, displays broad specificity," *Z Naturforsch [C]* 46: 585-90 (abstract)
	TR	Kwon O et al. (1996) "Menaquinone (vitamin K2) biosynthesis: overexpression, purification, and properties of o-succinylbenzoyl-coenzyme A synthetase from Escherichia coli," *J Bacteriol* 178: 6778-81.
	UR	Meganathan R et al. (1979) "Menaquinone (vitamin K2) biosynthesis: conversion of o-succinylbenzoic acid to 1,4-dihydroxy-2-naphthoic acid by Mycobacterium phlei enzymes," *J Bacteriol* 140: 92-8.
	VR	
	WR	
	XR	

Examiner | Date Considered:

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

1: Z Naturforsch [C]. 1991 Jul-Aug;46(7-8):585-90.

o-Succinylbenzoate: coenzyme A ligase, an enzyme involved in menaquinone (vitamin K2) biosynthesis, displays broad specificity.

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o-Succinylbenzoate: coenzyme A ligase, an enzyme involved in menaquinon biosynthesis, was purified from Mycobacterium phlei and characterized with respect to isoelectric point, molecular weight, pH optimum, temperature optimum and kinetic data. The enzyme hydrolyses ATP to AMP. The substrate and cofactor specificity of the enzyme was tested with analogues of o-succinylbenzoic acid, different nucleotides, thiols and divalent cations. The enzyme appears to possess broad specificity for substrates and cofactors.

PMID: 1663748 [PubMed - indexed for MEDLINE]

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0021-9193/96/$04.00+0

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Menaquinone (Vitamin K_2) Biosynthesis: Overexpression, Purification, and Properties of *o*-Succinylbenzoyl-Coenzyme A Synthetase from *Escherichia coli*

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The coenzyme A (CoA)- and ATP-dependent conversion of *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyric acid], to *o*-succinylbenzoyl-CoA is carried out by the enzyme *o*-succinylbenzoyl-CoA synthetase. *o*-Succinylbenzoyl-CoA is a key intermediate in the biosynthesis of menaquinone (vitamin K_2) in both gram-negative and gram-positive bacteria. The enzyme has been overexpressed and purified to homogeneity. The purified enzyme was found to have a native molecular mass of 185 kDa as determined by gel filtration column chromatography on Sephacryl S-200. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis established a subunit molecular mass of 49 kDa. Thus, the enzyme is a homotetramer. The enzyme showed a pH optimum of 7.5 to 8.0 and a temperature optimum of 30 to 40°C. The K_m values for OSB, ATP, and CoA were 16, 73.5, and 360 μM, respectively. Of the various metal ions tested, Mg^{2+} was found to be the most effective in stimulating the enzyme activity. Studies with substrate analogs showed that neither benzoic acid nor benzoylpropionic acid (succinylbenzene) is a substrate for the enzyme. Thus, it appears that both the benzoyl carboxyl group and the succinyl side chain are required for activation of the aliphatic carboxyl group.

Menaquinone (vitamin K_2) functions in several anaerobic electron transport systems of *Escherichia coli* (7). The pathway for the biosynthesis of menaquinone has been reviewed (8). To date, seven genes, designated *menA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G*, encoding the various enzymes of the pathway have been identified (8). An important step in the biosynthesis is the enzymatic conversion of the benzenoid aromatic compound *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyric acid] to the naphthalenoid aromatic compound 1,4-dihydroxy-2-naphthoic acid (DHNA). During this conversion, the bicyclic ring system makes its first appearance. The enzymatic cyclization of OSB to DHNA was shown to be dependent on the presence of ATP and coenzyme A (CoA), suggesting the formation of an OSB-CoA derivative as an intermediate (3, 9). Subsequently, by using cell extracts of *Mycobacterium phlei*, it was shown that two enzymatic activities, OSB-CoA synthetase and DHNA synthase, are required for this conversion. It was further demonstrated that the OSB-CoA synthetase forms a highly unstable intermediate, OSB-CoA, which was subsequently cyclized into DHNA by DHNA synthase (9). During the formation of OSB-CoA, the ATP was converted to AMP and PP_i. Consistent with these observations, two groups of mutants, designated *menE* and *menB*, lacking OSB-CoA synthetase and DHNA synthase, respectively, were identified (10, 14). These reactions, enzymes, and genes are summarized in Fig. 1. The gene encoding OSB-CoA synthetase has been cloned from *E. coli* (15), and its complete nucleotide sequence has been determined (12).

In this report, we describe the overexpression, purification, and properties of OSB-CoA synthetase from *E. coli*.

(A preliminary report of some of these findings has appeared [5]).

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MATERIALS AND METHODS

Materials. ATP, CoA, *E. coli* pyrophosphatase, DEAE Sephadex A-50, Reactive Blue 2 Sepharose CL-6B, protamine sulfate, trichloroacetic acid, ammonium molybdate, β-mercaptoethanol, *N,N,N′,N′*-tetramethylethylendiamine (TEMED), rifampin, and isopropyl-β-D-thiogalactopyranoside (IPTG) were products of Sigma Chemical Co., St. Louis, Mo. Low-molecular-weight protein standards were purchased from Bio-Rad Laboratories, Hercules, Calif. Centricon concentrators were from Amicon, Beverly, Mass. Ascorbic acid, sodium dodecyl sulfate (SDS), polyacrylamide, benzoic acid, phthalic acid, benzoylpropionic acid, *p*-coumaric acid, and *o*-acetylbenzoic acid were from Fisher Scientific Co., Pittsburgh, Pa. Restriction enzymes and the Altered Sites II in vitro mutagenesis kit were purchased from Promega, Madison, Wis. OSB was synthesized as described before (3).

Media and growth conditions. Cultures were routinely stored at −80°C in glycerated Luria-Bertani (LB) broth and grown on LB agar. Ampicillin, kanamycin, or tetracycline, when required, was added to the medium at a concentration of 50, 40, or 12.5 μg/ml, respectively, unless specified otherwise.

Construction of the overexpression plasmid. Plasmid pMS73 (12), containing the entire *menE* gene, was digested with *Eco*RI and *Hin*dIII. The resulting 2.4-kb fragment containing *menE* was subcloned into plasmid pAlter-1 (Promega) after restriction with the same enzymes, and the plasmid so formed was designated pME73. An *Nde*I restriction site was created near the ATG initiation codon of *menE* by site-directed mutagenesis with the Altered Sites II in vitro mutagenesis kit. An oligonucleotide primer spanning the region of the *menE* initiation codon was used for mutagenesis. The following sequence of the *menE* gene near the ATG codon, 5′TGGAGCGGTTGTTATGATCTTCTCTG3′, was altered by changing the two underlined bases to CA, and an *Nde*I site (CATATG) was introduced. The presence of the desired mutation was verified by restriction mapping, and the plasmid was designated pAE73. The expression vector pT7-7 (which contains the T7 promoter and ribosomal binding site [18]), was linearized with *Nde*I and *Hin*dIII and ligated with the 2.2-kb *Nde*I-*Hin*dIII fragment of pAE73, resulting in pME737. This overexpression plasmid was transformed into *E. coli* BL21 (DE3), which carries the T7 RNA polymerase gene under *lac* UV5 control (17).

Overexpression and preparation of cell extracts. A 5-ml overnight LB broth culture of *E. coli* BL21 (DE3) containing plasmid pME737 was inoculated into 500 ml of LB broth containing 0.2% glucose and 200 μg of ampicillin per ml and grown with shaking until the A_{600} reached 1.0. At this time, the T7 RNA polymerase was induced by addition of 0.4 mM IPTG, and after 30 min, rifampin was added at 200 μg/ml and growth continued for a further 3 h. The cells were harvested by centrifugation at 4,000 × *g* for 10 min. The pellet was washed by resuspension in 20 mM Tris-HCl buffer (pH 7.2), centrifuged, and stored frozen at −20°C. The frozen cells were suspended in a buffer of the same composition containing 10 mM β-mercaptoethanol (buffer A) at a ratio of 1 g/1.5 ml and passed through a French pressure cell at 12,000 lb/in^2. The extract was treated with DNase for 5 min at 37°C to reduce viscosity and centrifuged at 30,000 × *g* for 30 min, and the resulting supernatant was used for further purification.

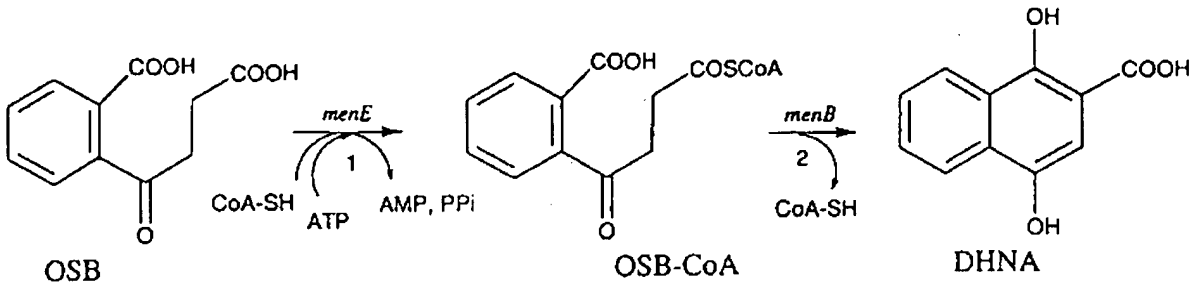

FIG. 1. The formation of OSB-CoA and its conversion to DHNA. 1, OSB-CoA synthetase; 2, DHNA synthase.

Protein determination. The protein concentrations were determined by the method of Bradford (2) with the Bio-Rad Laboratories protein assay kit. Bovine serum albumin was used as the standard.

Determination of pH optimum. For pHs 6.5 and 7.0, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer was used. From pH 7.0 to pH 8.5, 0.1 M Tris-HCl [Tris(hydroxymethyl)aminomethane] buffer was used. Enzyme assays were routinely performed in 0.1 M Tris-HCl buffer, pH 8.0.

Assay of enzyme activity. (i) By coupling with DHNA synthase. OSB-CoA, the product of the OSB-CoA synthetase reaction, is a highly unstable compound which spontaneously converts to OSB-spirodilactone in the absence of DHNA synthase (9, 12). Thus, it was assayed as accumulated DHNA following its conversion in the presence of excess DHNA synthase. The DHNA synthase was supplied in the form of an extract from *menE* mutant strain AN213 carrying plasmid pMS9 (13). The enzymatic incubation mixture, the assay conditions used, and the spectrophotofluorometric determination of the DHNA formed were as previously described (12, 13).

(ii) By coupling with pyrophosphatase. Based on the previous report that ATP is hydrolyzed to AMP and PP_i during the formation of OSB-CoA (9) (Fig. 1), a new, simple assay method for OSB-CoA synthetase was developed. The PP_i formed was converted to P_i by using *E. coli* pyrophosphatase, and the P_i was determined. The incubation mixture and the conditions were the same as those of the DHNA synthase coupled assay without the addition of AN213(pMS9) extract. The reaction was terminated by heating at 70°C. After termination, 1 U of *E. coli* pyrophosphatase was added to the reaction mixture and incubation was continued for 10 min at room temperature. The reaction was terminated by addition of 1 ml of 10% (wt/vol) trichloroacetic acid, and the precipitated protein was removed by centrifugation at 2,000 × *g*. The P_i was estimated as described by Chen et al. (4).

With the DHNA determination and phosphate determination methods described above, identical results were obtained. However, DHNA synthase has not been purified, necessitating the use of crude enzyme preparations in conjunction with homogeneous preparations of OSB-CoA synthetase. Hence, for all of the results reported here, the reaction was coupled with pyrophosphatase. Since the OSB-CoA synthetase in the strain has been amplified more than 3,000-fold, even in crude cell extracts, the phosphatase activity was very low. During the assay, a control containing all of the components except the substrate, OSB, was always included and the amount of phosphate formed was used to correct the experimental results. All of the assays were repeated at least three or four times, and the variation was less than 5%.

Protamine sulfate precipitation. The crude cell extract was acidified to pH 6.2 with 1% acetic acid. A 2% solution of protamine sulfate (adjusted to pH 6.2) was added dropwise with constant stirring until the volume increased by 40%, and the stirring was continued for another 30 min. The precipitate formed was removed by centrifugation at 30,000 × *g* for 30 min, and the clear supernatant was used for further purification.

DEAE Sephadex A-50 anion-exchange chromatography. A column (18 by 0.8 cm) of DEAE Sephadex A-50 was equilibrated with buffer A. Protamine sulfate supernatant (6.0 ml) was applied to the column, and the column was washed with 10 column volumes of buffer A until all of the unbound protein was removed. The bound enzyme was eluted with 160 ml of a linear gradient of 0 to 600 mM KCl in buffer A, and the A_{280} of the eluent was monitored. Fractions (2.0 ml) were collected, and the active fractions (no. 29 to 39) were pooled and concentrated with Centricon 30 concentrators (Amicon).

Blue Sepharose CL-6B affinity chromatography. The concentrated protein from the DEAE Sephadex A-50 column was applied to a Blue Sepharose CL-6B column (6 by 0.5 cm) equilibrated with buffer A and allowed to bind for 30 min. The column was washed with 10 volumes of buffer A to remove unbound protein. The enzyme was eluted with 160 ml of a linear gradient of 0 to 500 mM KCl in buffer A, and 2.2-ml fractions were collected. Fractions (no. 27 to 38) containing the enzyme activity were pooled and concentrated with Centricon 30 concentrators.

Spectroscopic methods. UV absorption spectra were recorded with a Perkin-Elmer lambda 4A spectrophotometer at 25°C.

SDS-PAGE. The purity of the enzyme preparation was monitored on SDS–12% polyacrylamide gel electrophoresis (PAGE) gels as described by Laemmli (6). The mini PROTEAN II cell apparatus of Bio-Rad Laboratories was used in accordance with the instructions of the manufacturer. The following low-molecular-weight Bio-Rad protein standards were used: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Native molecular mass determination. The native molecular mass of the enzyme was determined by Sephacryl S-200 gel filtration column chromatography as described in Sigma Chemical Co. technical bulletin GF-3. A column (1.5 by 100 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.2) and calibrated with the gel filtration kit of Sigma Chemical Co. The kit contained cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2,000 kDa). The molecular mass of OSB-CoA synthetase was determined from the calibration curve.

RESULTS

Purification of OSB-CoA synthetase. Overexpression strain BL21 (DE3) containing plasmid pME737 was grown and the enzyme was induced as described in Materials and Methods. Cell extracts of this strain produced 0.37 μmol of OSB-CoA min^{-1} mg of $protein^{-1}$ (Table 1). By using extracts of this overexpression strain as the starting point, the enzyme was purified by various methods (Table 1). Treatment of the clarified cell extract with protamine sulfate and removal of the precipitate by centrifugation resulted in approximately 1.4-fold purification of the enzyme in the supernatant, with greater than 60% recovery. The protamine sulfate supernatant was chromatographed on DEAE Sephadex A-50 anion-exchange resin, resulting in 3.5-fold purification with 53% recovery of the enzyme. The active fractions were combined and applied to a Blue Sepharose CL-6B affinity chromatography column, re-

TABLE 1. Purification of OSB-CoA synthetase of *E. coli*

Purification step	Total protein (mg)	Sp act (μmol min^{-1} mg of $protein^{-1}$)	Total activity (μmol min^{-1})	Yield (%)	Purification (fold)
Crude extract	390	0.37	144.3	100	1.0
Protamine sulfate	165	0.53	87.5	60.6	1.4
DEAE Sephadex A-50	58.5	1.31	76.7	53.2	3.5
Blue Sepharose CL-6B	22	3.20	70.4	48.8	8.6

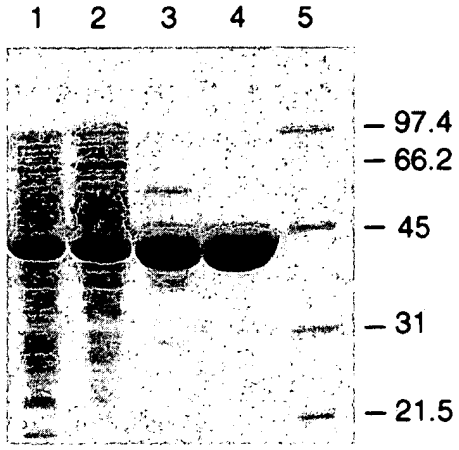

FIG. 2. SDS-PAGE analysis of protein fractions from various stages of purification. Proteins were separated on an SDS–12% PAGE gel and stained with Coomassie blue. Each lane contained 20 μg of protein. Lanes: 1, crude extract; 2, protamine sulfate fraction; 3, DEAE Sephadex A-50 fraction; 4, Blue Sepharose CL-6B fraction; 5, low-molecular-weight markers from Bio-Rad Laboratories. The values on the right are molecular masses in kilodaltons.

sulting in a ninefold-purified homogeneous preparation with a recovery of 49%. The purity of the enzyme during various stages of purification as determined by SDS-PAGE is shown in Fig. 2.

Molecular mass of the enzyme. The native molecular mass of the enzyme, as determined on a calibrated Sephacryl S-200 gel filtration column, was found to be 185 kDa. SDS-PAGE of the same sample gave a single band of 49 kDa.

Determination of pH and temperature optima. The pH optimum of the enzyme was determined in the range of 6.5 to 8.5. The enzyme exhibited optimum activity at pHs 7.5 to 8.0. At a pH of 7.0, the activity decreased only slightly. However, the activity decreased sharply at pHs of 6.5 and 8.5 to about 55 and 80%, respectively, of that obtained at the optimum pH (data not shown). The enzyme showed maximum activity in the temperature range of 30 to 40°C (data not shown).

Determination of kinetic constants. The OSB-CoA synthetase activity increased with increasing concentrations of the substrates OSB, ATP, and CoA, and typical hyperbolic curves were obtained (data not shown). The apparent K_m and V_{max} were determined for each of the substrates by using double-reciprocal plots. The K_m values for OSB, ATP, and CoA were 16, 73.5, and 360 μM, respectively, and the corresponding V_{max} values were 2, 2.4, and 4.3 μmol min^{-1} mg of $protein^{-1}$.

Activity with substrate analogs. Various benzenoid substrate analogs, *o*-malonylbenzoic acid, benzoylpropionic acid, benzoic acid, *o*-acetylbenzoic acid, phthalic acid, and *p*-coumaric acid, were tested for enzyme activity and were found to be inactive (data not shown).

Absorption spectra of the enzyme. The absorption spectrum of the purified enzyme was recorded at wavelengths of 250 to 700 nm. It showed a single absorption peak at 278 nm (data not shown).

Metal ion requirement for enzyme activity. The metal ion requirement for the enzyme activity was determined at 0.5, 1.0, and 5.0 mM concentrations. As seen in Table 2, the presence of Na^+, K^+, Ca^{2+}, or Zn^{2+} resulted in approximately twofold higher activity than the control at all of the concentrations tested. Addition of Co^{2+} or Mn^{2+} increased the activity approximately fourfold at 0.5 and 1.0 mM, while at 5 mM, the increase was only twofold. Maximum stimulation of sixfold was observed at 1 mM Mg^{2+}, while at 0.5 and 5.0 mM, the increases were only five- and fourfold, respectively. In the presence of Fe^{2+} and Hg^{2+}, the enzyme was inhibited.

TABLE 2. Effects of various metal ions on OSB-CoA synthetase activity[a]

Metal ion	Sp act (μmol min^{-1} mg of $protein^{-1}$) at ion concn of:			
	0 mM	0.5 mM	1 mM	5 mM
None	0.53			
Na^+		0.83	0.83	0.83
K^+		0.83	0.90	1.0
Mg^{2+}		2.65	2.92	2.2
Ca^{2+}		1.0	1.0	ND[b]
Zn^{2+}		0.92	1.26	ND
Co^{2+}		1.75	2.25	1.07
Mn^{2+}		1.87	2.02	1.14
Fe^{2+}		0.29	0.43	ND
Hg^{2+}		0.18	0.19	ND

[a] For each assay, 1.0 μg of the enzyme was used. Each value is the average of at least four determinations.
[b] ND, not determined due to precipitation.

Since the enzyme showed maximum activity with Mg^{2+}, the effect of various concentrations of this metal on the enzyme activity was determined (Fig. 3). Very little activity was observed at concentrations of 0.1 to 10 μM, and the activity increased sharply from 25 μM, reaching a maximum at 1 mM. At concentrations above 1 mM, the enzyme was inhibited.

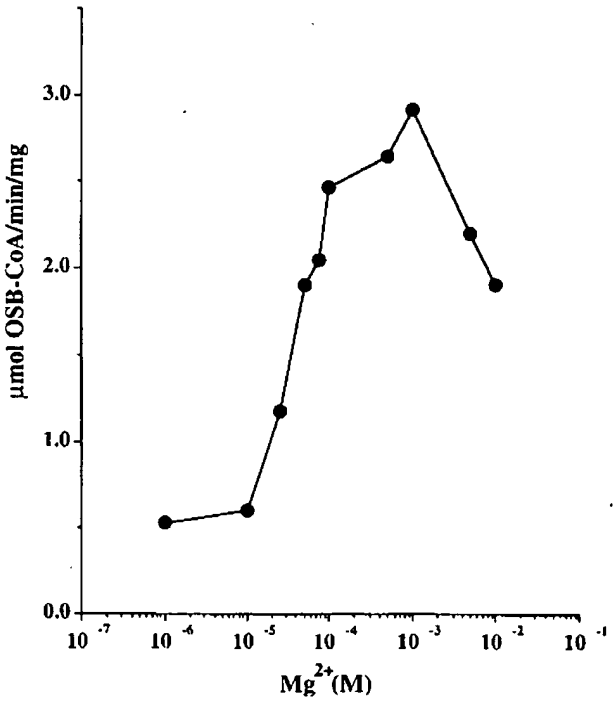

FIG. 3. Effect of various concentrations of Mg^{2+} on OSB-CoA synthetase activity. Enzyme activity was measured as described in Materials and Methods, and the Mg^{2+} concentration was varied from 10^{-7} to 10^{-2} M as indicated.

DISCUSSION

Conversion of the benzenoid OSB to the naphthalenoid DHNA requires the elimination of water and cyclization. This process is initiated by the enzyme OSB-CoA synthetase, resulting in the formation of OSB-CoA as an intermediate. The presence of OSB-CoA synthetase and DHNA synthase has been reported in the gram-negative bacterium *E. coli* (14) and a number of gram-positive organisms, such as *Bacillus subtilis* (10), *Micrococcus luteus* (11), and *M. phlei* (9). Since the enzymes involved in menaquinone biosynthesis are present at extremely low levels, early attempts at purification and characterization of the enzyme met with very limited success. Partial purification of OSB-CoA synthetase to near homogeneity from *M. phlei*, an organism which shows, in crude extracts, a specific activity 1.5 times that of *E. coli*, was achieved (1). However, the enzyme was not characterized further due to the small quantities available. Subsequently, a 200-fold partial purification and characterization of the enzyme from *M. phlei* were reported (16).

The activity of OSB-CoA synthetase in the wild-type strain of *E. coli* is about 0.12 nmol min^{-1} mg of $protein^{-1}$, which is extremely low for meaningful purification and characterization of the enzyme. Recently, we reported the complete nucleotide sequence of the *menE* gene encoding the OSB-CoA synthetase in *E. coli* (12). This information enabled us to overexpress this protein. The overexpressed strain had an activity of 370 nmol min^{-1} mg of $protein^{-1}$, which is about 3,000-fold higher than that of the wild-type strain and constituted about 11% of the total cellular protein. Extracts from this strain were used to purify the protein ninefold, resulting in a homogeneous preparation with a specific activity of 3.2 μmol min^{-1} mg of $protein^{-1}$. It was calculated that this level of activity is over 25,000-fold higher than that of the wild-type strain and allowed further characterization of the enzyme. The product of the reaction, OSB-CoA, is a highly unstable intermediate which cannot be assayed directly (9). We developed a simple colorimetric assay by determining PP_i after its hydrolysis to P_i by pyrophosphatase. This assay is based on previous studies establishing the hydrolysis of ATP to AMP and PP_i during the formation of OSB-CoA (9).

The purified enzyme had a molecular mass of 49 kDa as determined by SDS-PAGE, which is in agreement with the calculated molecular mass of 50.2 kDa as determined by sequence analysis (12). Since the native molecular mass as determined by gel filtration was 185 kDa, it appears that the enzyme is a homotetramer. The *M. phlei* enzyme, in contrast, has been reported to have a native molecular mass of 28 kDa (16). The K_m values for OSB, ATP, and CoA were found to be 16, 73.5, and 360 μM, respectively, while the *M. phlei* enzyme had K_m values of 148.1 and 16.5 μM for OSB and CoA, respectively. Sieweke and Leistner were unable to determine the K_m for ATP (16).

Further, it was reported for the *M. phlei* enzyme that the substrate analogs *o*-malonylbenzoic acid and benzoylpropionic acid had 11.6 and 21.6% of the activity, respectively, of that obtained with OSB, while benzoic acid, *o*-acetylbenzoic acid, phthalic acid, and *p*-coumaric acid were inactive (16). All of the above substrate analogs, except for *o*-malonylbenzoic acid, were tested and found to be inactive for the *E. coli* enzyme. Since neither benzoic acid nor benzoylpropionic acid (succinylbenzene) served as a substrate for the enzyme, it appears that both the benzoyl carboxyl group and the succinyl side chain are required for activation of the aliphatic carboxyl group.

While the partial purification and properties of OSB-CoA synthetase from *M. phlei* have been reported previously (1, 16), this is the first report of the purification of the enzyme to homogeneity. To further clarify the catalytic and regulatory properties of the enzyme, chemical modification and site-directed mutagenesis studies are in progress.

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Menaquinone (Vitamin K_2) Biosynthesis: Conversion of *o*-Succinylbenzoic Acid to 1,4-Dihydroxy-2-Naphthoic Acid by *Mycobacterium phlei* Enzymes

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The coenzyme A (CoA) and adenosine 5'-triphosphate-dependent conversion of *o*-succinylbenzoic acid (4-[2'-carboxyphenyl]-4-oxobutyric acid) to 1,4-dihydroxy-2-naphthoic acid is an important step in menaquinone (vitamin K_2) biosynthesis. Cell-free extracts catalyzing this conversion, obtained from *Mycobacterium phlei*, were separated into three protein fractions by treatment with protamine sulfate. The second fraction (fraction B) and the supernatant (fraction S) alone did not catalyze dihydroxynaphthoate formation, but did so in combination. All of the results were consistent with the formation of an unstable intermediate, likely an *o*-succinylbenzoyl-CoA compound, by the action of fraction S. Adenosine 5'-triphosphate was required in this reaction and adenosine 5'-monophosphate was formed. This enzyme activity was termed *o*-succinylbenzoyl-CoA synthetase: the enzyme showed a marked stability to 0.1 N hydrochloric acid. The presumed *o*-succinylbenzoyl-CoA derivative was rather unstable; under a variety of conditions, it was converted to a spirodilactone form of *o*-succinylbenzoate. Fraction B contained an enzyme, termed naphthoate synthase, which converted the *o*-succinylbenzoyl-CoA derivative to 1,4-dihydroxy-2-naphthoate.

The first aromatic compound so far identified as an intermediate in the biosynthetic pathway to menaquinone (vitamin K_2) is the benzenoid compound *o*-succinylbenzoic acid (OSB; 4-[2'-carboxyphenyl]-4-oxobutyric acid). An important step in the pathway is the formation of the bicyclic aromatic ring system; this is achieved by a cyclization of OSB to the naphthalenoid compound 1,4-dihydroxy-2-naphthoic acid (DHNA; see references 2 and 3 for reviews). Cell-free extracts catalyzing the conversion OSB → DHNA have been obtained from *Escherichia coli* and *Mycobacterium phlei*; the formation of DHNA was observed to be dependent on the presence of both coenzyme A (CoA) and ATP (5, 9). Attempted purification of this "naphthoate synthetase" activity was not very successful, only a fivefold increase in specific activity being obtained. The work reported in this paper shows that the cell-free preparations from *M. phlei* contain at least two separate enzymes. One apparently catalyzes the conversion of OSB to a CoA derivative; it will be termed *o*-succinylbenzoyl-CoA synthetase, abbreviated to OSB-CoA synthetase. The second enzyme, which is believed to be responsible for the actual cyclization, will be termed 1,4-dihydroxy-2-naphthoate synthase, abbreviated to DHNA synthase. The preparations previously called naphthoate synthetase are thus a mixture of at least two enzymes.

A preliminary report of these studies has appeared (R. Meganathan and R. Bentley, Fed. Proc. **38**:315, 1979).

MATERIALS AND METHODS

Preparations of buffer and protamine sulfate solutions. Throughout this work, MOPS (3-[*N*-morpholino]propanesulfonic acid) plus KOH mixtures were used for buffers. Buffer A was prepared by adjusting a 0.02 M solution of MOPS in 20% (vol/vol) aqueous dimethyl sulfoxide to pH 6.9 by addition of 20% KOH; 360 μl of 2-mercaptoethanol (5 mM) was then added. Buffer B was 0.05 M MOPS in water, adjusted to pH 6.9, and containing the same amount of mercaptoethanol. Buffer C was prepared exactly as buffer A, but using a 20% (vol/vol) solution of glycerol as the solvent. A 2% protamine sulfate solution was prepared in buffer A; to facilitate solution, the operation was carried out at 37°C. Before use, a 25-ml portion was adjusted to pH 6.9 with 20% KOH and was vacuum filtered through Whatman no. 1 filter paper.

Preparation of cell-free extracts. *M. phlei* ATCC 354 was maintained, grown, and harvested as described earlier (9). After the cells were washed the cell paste was kept frozen at −20°C. Active extracts have been obtained with cells stored for as long as 2 months. The cell paste, typically 20 g, was thawed by suspension in 30 ml of buffer A. This mixture, at a temperature of 0°C, was passed through a French

pressure cell, using a pressure of 10,000 to 12,000 lb/in^2; the operation was carried out in the cold. To the viscous extract was added DNase (approximately 1 mg, 1,200 U/mg), and the mixture was then allowed to incubate at 30°C for 5 min. This treatment was followed by centrifugation at 12,000 × *g* and 4°C for 10 min in a Sorvall RC 2 centrifuge to remove unbroken cells and cell debris. The supernatant solution was again centrifuged at 17,300 × *g* for a further 10 min.

Assay of DHNA. DHNA formation was measured spectrophotofluorometrically as previously described (9). The incubation mixtures contained, in micromoles: OSB, 0.25; ATP, 4.8; CoA, 0.5; $MgCl_2$, 20. In each case, the final volume was made up to 3 ml with buffer B, and the mixtures were incubated at 30°C for 30 min. The specific activity of enzyme systems forming DHNA will be expressed as nanomoles of DHNA per 30 min per milligram of protein or as nanomoles of DHNA per 30 min per tube.

General. Cell extracts were assayed for protein by the method of Bradford (4), using the reagents and protein standard supplied by Bio-Rad Laboratories. ATP, CoA, and protamine sulfate were from Sigma Chemical Co., MOPS was from Calbiochem, and DNase was from Worthington Biochemicals Corp. All other reagents were of the highest quality available, and all solvents were redistilled before use. [*U*-^{14}C]-ATP was obtained from Amersham Corp., and [2-^{14}C]pyruvate, a product of CEA, Saclay, France, was obtained from Research Products International Corp. The polyethyleneimine thin-layer plates were a product of Macherey-Nagel Co. OSB, OSB spirodilactone, and DHNA were synthesized as previously described (5, 9). Although described here as OSB spirodilactone, and although alkaline hydrolysis of this material yields OSB, the compound is more accurately called the spirodilactone of 4-(2′-carboxyphenyl)-4,4-dihydroxybutyric acid. [2-^{14}C]OSB was synthesized by the use of [2-^{14}C]pyruvate in the following reaction sequence (P. Dansette, Doctor of Science thesis, University of Paris-South, Centre D'Orsay, France, 1972). Reaction of 2-carboxybenzaldehyde with pyruvate gave 2-carboxybenzylidene pyruvate, which was directly reduced with sodium borohydride to 2-carboxybenzylidene lactate; acid isomerization (6) of the lactate yielded OSB. Spectrophotofluorometric measurements were made with the Aminco-Bowman instrument (no. 4-8202); the excitation wavelength was 370 nm and the emission wavelength was 430 nm (9). Radioactivity on thin-layer chromatograms was detected with a Packard model 7201 radiochromatogram scanner.

RESULTS

Fractionation of the crude extract. Early attempts to purify the naphthoate synthetase of two microorganisms by using columns of Sephadex G-150 (5) or Sepharose CL-6B (9) achieved only modest success. A new approach, adopted in the present work, was to carry out a fractionation with protamine sulfate. Since the naphthoate synthetase had appeared to be rather unstable in the presence of reagents such as ammonium sulfate, the addition of dimethyl sulfoxide to the solutions was investigated. Evidence for a fractionation was obtained, and dimethyl sulfoxide has been incorporated routinely for the extraction and precipitation. As noted later, its exact role is unclear. A typical protocol is as follows. The crude cell-free extract from *M. phlei*, obtained from the French press treatment, was diluted with buffer A so that the protein concentration was 24 mg/ml. To 20 ml of this extract at 0°C was added 9 ml of the filtered protamine sulfate solution at 37°C, slowly and with continuous stirring. The heavy precipitate which formed was separated by centrifugation at 12,100 × *g* and 4°C for 10 min. The precipitate was then dissolved in 5 ml of ice-cold 20% saturated ammonium sulfate solution which had been prepared with buffer A. This solution was termed fraction A.

The supernatant solution remaining from the centrifugation was next treated with a further 11-ml portion of protamine sulfate solution, as above, with stirring. When the addition was complete, the mixture was allowed to stand on ice for 20 min. A precipitate formed and was removed by centrifugation at 12,100 × *g* and 4°C for 10 min. This precipitate was dissolved in ammonium sulfate solution as described for the first precipitate, and the resulting solution was designated fraction B. The remaining clear supernatant after removing these two precipitates was termed fraction S. It was not possible to precipitate any further amount of protein from this supernatant fraction by addition of more protamine sulfate or ammonium sulfate, unless the dimethyl sulfoxide was first removed by dialysis. The enzyme remaining in solution under these fractionation conditions will subsequently be identified as an *o*-succinylbenzoyl-CoA synthetase, whereas that precipitating in fraction B will be identified as a dihydroxynaphthoate synthase.

The amount of protamine sulfate solution required for the fractionation depends to some extent on which batch of protamine sulfate was used and on the individual cell extracts. The quantities described above were necessary with lot P23B-258 of the Sigma Chemical Co. material. The exact amount needed was determined on a trial-and-error basis with small portions of each extract.

The crude extract from *M. phlei* catalyzed the formation of DHNA from OSB (Table 1); the specific activity of the system was 1.78 nmol of DHNA formed/30 min per mg of protein. When the three fractions just described were similarly assayed for DHNA production, none of them alone showed any activity. However, DHNA synthesis was obtained on combining fractions

A, B, and S. Furthermore, experiments with mixtures of two fractions showed that DHNA was formed using the combination of fraction B and fraction S (see Table 1).

Fraction S contains an acid-stable enzyme activity. In view of the absolute requirement for the participation of CoA and ATP, it appeared reasonable to assume that one fraction was responsible for producing a CoA derivative of OSB, while the other was responsible for cyclizing this intermediate to DHNA. The following experiments were carried out to determine whether any actual accumulation of an intermediate could be detected. It was reasoned that if an intermediate did exist, separate incubation of either fraction S or fraction B with OSB, CoA, ATP, and Mg^{2+} would lead to its accumulation. After acid treatment to denature the first enzyme, addition of fraction B to the tube originally containing fraction S (or vice versa) would yield DHNA for only one of the two possible sequences (fraction S first, then B; or fraction B first, then S). Such experiments were performed, using a 30-min incubation period for both phases and 0.1 N HCl (final concentration) to denature protein after the first phase: the solutions were neutralized before addition of the second enzyme. The results seemed to indicate (see Table 2) that DHNA was synthesized only when the sequence was fraction S followed by fraction B. However, to provide controls, portions of the two fractions had been denatured with acid immediately after the addition of the substrates. The acidified solutions were then neutralized and treated with the other fraction. Surprisingly, the "control" experiment with fraction S as the first component formed almost as much DHNA (on subsequent treatment with fraction B; see Table 2) as that which was not treated with acid until after the 30-min initial incubation period. In further experiments of this kind, the acid concentration used for enzyme denaturation after the first incubation period was raised to 0.5 N. Under these conditions, the fraction S controls formed essentially no DHNA. These experiments, therefore, lead to the conclusion that the enzyme of fraction S has a marked stability to 0.1 N HCl but is denatured by brief exposure to 0.5 N HCl.

As for the original objective of these experi-

TABLE 1. *DHNA formation by protamine fractions of M. phlei extracts*

Component	Protein (mg/tube)	DHNA formed[a] (nmol/tube per 30 min)
Crude extract	5.9	10.5
Crude extract minus OSB	5.9	<0.2[b]
Fraction A	5.0	<0.2[b]
Fraction B	4.1	<0.2[b]
Fraction S	1.23	<0.2[b]
Fractions A + B + S	5.0 + 4.1 + 1.23	10.1
Fractions A + B	5.0 + 4.1	<0.2[b]
Fractions A + S	5.0 + 1.23	<0.2[b]
Fractions B + S	4.1 + 1.23	12.3

[a] DHNA formation was assayed spectrophotofluorometrically (9); the assay mixture is described in the text.

[b] At these low levels, the assay lacks precision.

TABLE 2. *Effect of acid on the enzyme activities of fractions B and S*

Tube no.	Fraction used in first incubation[a] (mg)		Reaction time (min)	Acid to denature (N)	Fraction used in second incubation (mg)		DHNA formed (nmol/tube)
	B	S			B	S	
1	4.1	—[b]	0	0.1	—	1.23	<0.1[c]
2	—	1.23	0	0.1	4.1	—	6.4
3	4.1	—	30	0.1	—	1.23	<0.2[c]
4	—	1.23	30	0.1	4.1	—	8.1
5	—	1.23	0	0.5	4.1	—	0.5[d]
6	—	1.23	30	0.5	4.1	—	1.8

[a] All tubes initially contained the amounts of OSB, CoA, ATP, and Mg^{2+} used in the routine assay and the indicated amount of either fraction B or fraction S. The contents of tubes 1 and 2 were treated with 3 N HCl at zero time to yield a final concentration of 0.1 N. They were then neutralized to pH 6.9 with 5% KOH. The tube originally containing fraction B now received the indicated addition of fraction S and vice versa. The production of DHNA was then determined after a further 30 min of incubation. In the case of tubes 3 and 4, a similar approach was used except that the initial addition of acid was not made until after a 30-min incubation. The experiments reported for tubes 5 and 6 were carried out at a different time from the others but are combined in the table for convenience. They differ only in the use of 0.5 N HCl to denature after the first incubation.

[b] —, No addition of indicated fraction.

[c] At these low levels, the assay lacks precision.

[d] A control without any treatment carried out at the time of these experiments gave a DHNA yield of 8.8 nmol/tube; this yield is comparable to that obtained in tube 4.

ments, only a tentative conclusion was possible. When fraction S was incubated with the appropriate additions for 30 min and then acidified with acid, neutralized, and further incubated with fraction B, the amount of DHNA formed was slightly more than in the control. This was true for treatment with both 0.1 N HCl (8.1 versus 6.4 nmol; see Table 2, tubes 4 and 2) and 0.5 N HCl (1.8 versus 0.5 nmol; see Table 2, tubes 6 and 5). Although the differences are small (and measurements in the lower range of the assay are particularly subject to variation), they were observed consistently. If valid, these results indicate that any intermediate has a limited stability and does not accumulate to a significant extent. They also suggest that the intermediate is formed by fraction S (OSB-CoA synthetase) and is cyclized by fraction B (DHNA synthase). Attempts to increase the production of intermediate by increasing the incubation time were unsuccessful.

Other evidence for the OSB-CoA intermediate. Since the formation of acyl CoA derivatives is usually accompanied by the conversion of ATP to AMP, the reaction was studied in the presence of [*U*-^{14}C]ATP. In this work, the protamine sulfate supernatant (fraction S) was further purified. The supernatant, 40 ml, was first dialyzed for 4 h at 4°C against 50 times its volume of buffer C. Finely ground ammonium sulfate was then added to 75% saturation at 0°C, and the mixture was centrifuged at 12,100 × *g* and 4°C for 10 min. The precipitate was dissolved in 4 ml of buffer C, and the solution was treated with 3 N HCl to yield a final concentration of 0.1 N. This mixture was dialyzed against 200 times its volume of buffer C for 1 h; the buffer solution was then changed, and dialysis was continued for 1 h. During dialysis, a white precipitate formed. The precipitate was removed by centrifugation at 12,000 × *g* for 10 min and was discarded. When the supernatant was assayed in the usual way, about a 50-fold increase in specific activity was observed. In some cases, the majority of the enzyme remained in the precipitate; whether it was present in supernatant or precipitate apparently depended on the batch of protamine sulfate used in the fractionation.

For the experiment with [*U*-^{14}C]ATP shown in Fig. 1, a portion of the purified supernatant just discussed (10 μl, 13 μg of protein) was incubated with [*U*-^{14}C]ATP (total cpm, 149,000; specific radioactivity, 540 mCi/mmol) and the following amounts, in micromoles, of other components: OSB, 0.025; CoA, 0.05; Mg^{2+}, 2. The total volume was 50 μl, a capillary tube being used. Incubation was at room temperature for 1 h. A control tube contained all components except OSB. After incubation, the contents of the tubes were streaked on polyethyleneimine thin-layer sheets, and the chromatograms were developed in 1 M LiCl solution in open vessels until the solvent front had moved a distance of approximately 15 cm (10). The dried sheets were examined in the radiochromatogram scanner. The control material formed two prominent radioactive peaks of almost equal size, at the po-

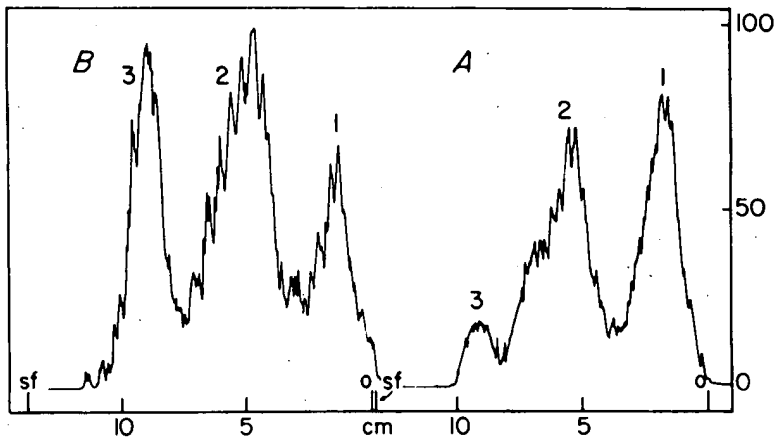

FIG. 1. *Production of AMP by action of OSB-CoA synthetase. After the incubation described in the text and thin-layer chromatography, the sheets were scanned in the radiochromatogram scanner. The ordinate shows percentage of full-scale deflection. The instrument settings were as follows: time constant, 10 s; range, 3 × 10^3 cpm; speed, 0.5 cm/min. In the experiment shown in (A), OSB had been omitted. The experiment shown in (B) contained all components as described in the text. o, Origin; sf, solvent front. The peaks corresponded to the positions of standard samples and are identified by numbers: 1 = ATP; 2 = ADP; 3 = AMP.*

sitions expected for ATP and ADP; the control showed only a very small peak of radioactivity at the R_f value associated with AMP (Fig. 1A). By contrast, in the solution containing all of the components, the peak of radioactivity for AMP was at least as large as that for ADP and the ATP peak was much diminished (see Fig. 1B). Thus, it appears that the product of the reaction is AMP; the presence of ADP is ascribed to the presence of an ATPase activity. The formation of AMP is consistent with the proposed role for an OSB-CoA intermediate.

To obtain further information as to the fate of OSB in these systems, [2-^{14}C]OSB was incubated with fraction S in the usual way in the presence of CoA, ATP, and Mg^{2+}. On extraction of organic materials and thin-layer chromatography, one radioactive spot was observed in addition to that for OSB itself (see Fig. 2A); the position of this spot corresponded exactly with that of authentic spirodilactone of OSB. When a sample of DHNA synthase (fraction B) was added to the incubation with [2-^{14}C]OSB, an additional radioactive peak was produced which was identified as DHNA (see Fig. 2B); at the same time, the formation of the spirodilactone of OSB was much decreased. Thus, these experiments provided further evidence for the lability of the presumed intermediate and established that it was easily converted to the spirodilactone form of OSB. However, in the presence of DHNA synthase, this alternative pathway was suppressed.

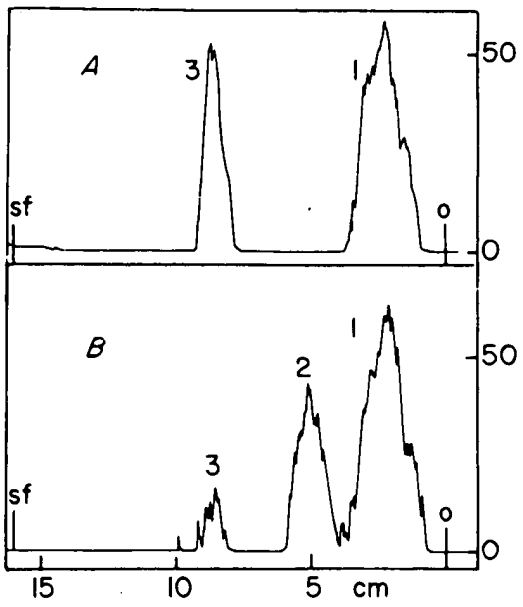

FIG. 2. *Formation of OSB spirodilactone by extracts from M. phlei. These experiments were carried out with [2-^{14}C]OSB with a specific radioactivity of 25 mCi/mmol, and in each case approximately 100,000 cpm were added. Other additions were as described in the text, except that 0.02 and 0.1 M potassium phosphate were used in place of MOPS for buffers A and B. Incubations were carried out for 30 min at 30°C. For the experiment shown in (A), 1.0 mg of fraction S was used; for that in (B), both fraction S (1.0 mg) and 11 U of fraction B were present. After incubation, the contents of the tubes were extracted with 6 ml of a mixture of acetone-benzene-concentrated HCl, 100:100:1. Four milliliters of the organic phase was withdrawn, mixed with 100 μg each of OSB, DHNA, and spirodilactone, and evaporated to dryness in a rotary evaporator. The samples were redissolved in 500 μl of ethyl acetate and then spotted on Analtech Silica Gel GF thin-layer plates. They were developed in the following solvent: chloroform-ethyl acetate-formic acid, 135:20:1.5. After development, the plates were scanned for radioactivity in the radiochromatogram scanner. The ordinate of the figure shows percentage of full-scale deflection; the instrument settings were: time constant, 10 s; range, 3 × 10^3 cpm; speed, 1 cm/min. o, Origin; sf, solvent front. The peaks corresponded with the positions of standard samples and are identified by numbers: 1 = OSB; 2 = DHNA; 3 = spirodilactone of OSB.*

DISCUSSION

The results of this work demonstrate unequivocally that the naphthoate synthetase of *M. phlei* can be fractionated into two components with the aid of protamine sulfate. The presence of dimethyl sulfoxide is critical to the fractionation process; this solvent apparently holds one of the enzymes in solution while allowing precipitation of the other. The following results support our earlier contention (5, 9) that a CoA derivative of OSB is required for the enzymatic cyclization of OSB to DHNA, although the apparent instability of this intermediate has frustrated attempts to characterize it by more direct means. (i) The separation of two protein fractions, both of which are required for DHNA synthesis, argues strongly for an intermediate. All of the evidence reported here indicates that the supernatant fraction, fraction S, is responsible for synthesis of the OSB-CoA compound. (ii) The reaction catalyzed by fraction S requires ATP in addition to CoA and OSB. The ATP is cleaved to AMP, and the reaction appears to be typical of the various acyl CoA synthetases (1). Fraction S is, therefore, identified as OSB-CoA synthetase, and the reaction is represented as follows:

$$\text{OSB} + \text{ATP} + \text{CoA-SH} \xrightarrow[\text{synthetase, } Mg^{2+}]{\text{OSB-CoA}} \text{OSB-S-CoA} + \text{AMP} + \text{PP}_i$$

(iii) In the absence of fraction B, the major

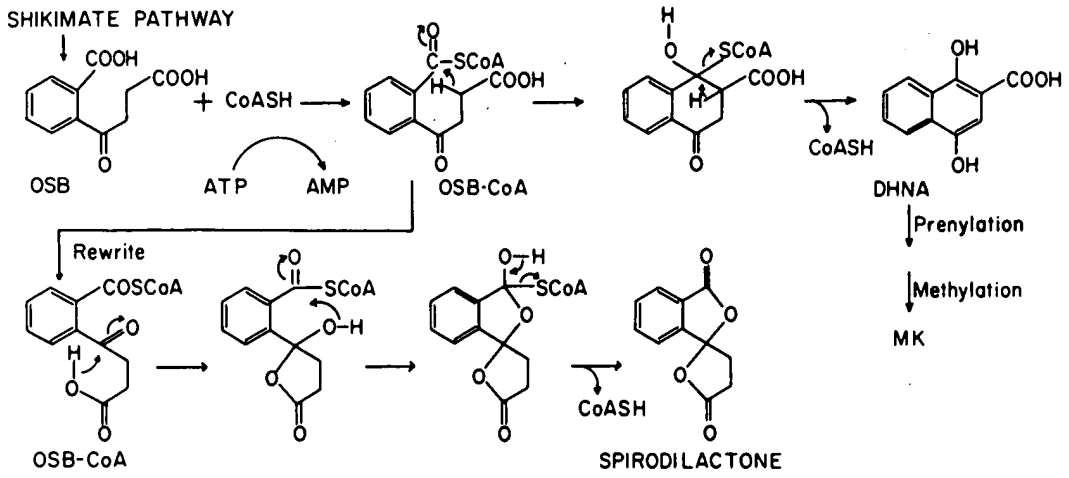

FIG. 3. *Role of the CoA derivative of OSB in formation of menaquinone (MK) and the spirodilactone. OSB itself is formed from chorismate and ultimately shikimate, as implied by the expression "shikimate pathway."*

product of the action of fraction S on OSB is the spirodilactone; when fraction B is added, the formation of spirodilactone is considerably diminished, and DHNA production occurs. These results also argue for the formation of an intermediate, which decomposes to spirodilactone in the absence of fraction B. Fraction B is, therefore, assigned a role in the cyclization of the intermediate and is named DHNA synthase; the reaction is represented as follows.

$$\text{OSB-CoA} \xrightarrow{\text{DHNA synthase}} \text{DHNA} + \text{CoA-SH}$$

The alternative possibilities for the OSB-CoA intermediate, and their relation to the menaquinone biosynthetic pathway, may be summarized by the reactions shown in Fig. 3. Although the difficulties of working with the CoA intermediate have not yet allowed us to determine its constitution and structure, we have proposed (5, 9) that the most likely location for the CoA residue is on the 2'-carboxyl group (the aromatic carboxyl) of OSB. The formation of the spirodilactone appears to represent a nonenzymatic decomposition of the reactive OSB-CoA intermediate. It is known that the spirodilactone is not as efficient a precursor for the plant naphthoquinone, lawsone, as is OSB itself (7), and there does not seem to be any need to assign it a role in menaquinone biosynthesis.

After our work with *E. coli* and *M. phlei* preparations, Hutson and Threlfall searched for naphthoate synthetase activity in extracts of *Micrococcus luteus* (8). They were unable to show the formation of DHNA but did observe a synthesis of OSB spirodilactone which was dependent on the presence of CoA and ATP. They also have assumed the formation of the OSB-CoA derivative, as suggested by us, with the CoA residue on the aromatic carboxyl. In view of the results reported here, it appears likely that their preparations did not contain any DHNA synthase, possibly because spray-dried cells were used as the starting material. As our work indicates, this enzyme is relatively unstable, particularly in comparison with the OSB-CoA synthetase.

The OSB-CoA synthetase, even in the absence of dimethyl sulfoxide, shows a marked stability to 0.1 N HCl. It was possible to obtain a 50-fold purification of the enzyme by taking advantage of this property. This degree of purification is very encouraging, especially since the maximum purification of the naphthoate synthetase was at most fivefold.

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